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(54) Title: MODIFIED GP100 AND USES THEREOF

(57) Abstract: A gp100 molecule useful in modulating the immune system is described. In particular what is described is a nu-  
cleotide sequence for a gp100 which has been modified so as to be useful in modulating the immune system. The isolated nucleic acid  
sequence is described and referred to as gp100M. The corresponding protein, referred to as gp100M, is also described. Applications  
of the molecules as vaccines are described.

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**Title: MODIFIED GP100 AND USES THEREOF**

This application claims the benefit of United States application serial no. 60/160,879, filed on October 22, 1999 and United States application serial no. 60/223,325 filed on August 7, 2000, both of which are incorporated by reference herein.

**FIELD OF THE INVENTION**

The present invention is in the field of immunotherapy and relates to the construct of novel forms of gp100 suitable for the generation of immune responses *in vivo*.

**BACKGROUND OF THE INVENTION**

The incidence and mortality of cutaneous malignant melanoma have risen dramatically over the past several decades (Liu, T. et al. (1996) *Surg Clin North Am* 76:1205; Gloster, H.M. et al. (1996) *Dermatol Surg* 22:217). At present, the estimated lifetime risk for an American of developing melanoma is approximately 1 in 90 (Rigel, D.S. et al. (1979) *Mayo Clin Proc* 72:367). While most early stage melanomas can be treated successfully by a simple surgical excision (Greenstein D.S. et al. (1995) *Dermatol Surg* 21:927; Whooley, B.P. et al. (1995) *Dermatol Surg* 4:187; Urist, M.M. et al. (1996) *Ann Rev Med* 47:211), patients with advanced disease are rarely cured even with aggressive chemotherapy and/or immunotherapy (Falkson, C.I. et al. (1995) *Anticancer Drugs* 6:709).

Although melanoma can present as an aggressive primary lesion that metastasizes within weeks, its development typically takes place over a period of several months to years and progresses through a series of distinct pathological stages. The earliest stage is the radial growth phase (RGP) melanoma that starts as an intradermal neoplasm (melanoma *in situ*). RGP melanomas may remain relatively quiescent for months or even years and are generally cured by simple excision. Eventually, most RGP melanomas develop a component of vertical growth (vertical growth phase melanoma, VGP), which denotes a more aggressive tumor less likely to be cured by simple excision. Metastatic spread is

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the final stage in tumor progression and is indicative of a very poor outcome (Reintgen, D. (1997) *Ann Surg* 225:1).

The most important prognostic factor in determining survival in a patient with primary melanoma is the depth of the lesion. While the five-year survival for patients with tumours of  $\leq 0.75$  mm in thickness is approximately 96%, individuals with tumours  $> 4.5$  mm in depth have a five year survival of only approximately 38% (Whooley, B.P. et al. (1995) *Dermatol Surg* 4:187; Urist, M.M. et al. (1996) *Ann Rev Med* 47:211). Since the development of a VGP melanoma from its RGP precursor generally takes at least several months, a "window of time" exists within which surgical excision can have a dramatic effect on patient outcome. By extension, it is reasonable to propose that novel attempts to deal with residual disease would be most successful at this time.

Several lines of evidence suggest that manipulation of immune responses against melanoma may be therapeutic: 1. Clinical observations of spontaneous regression of metastatic melanoma may be caused by an anti-melanoma immune response (Nelson, C.A. et al. (1976) *Natl Cancer Inst Monogr* 44:145). 2. Regression of metastatic melanoma has also been observed in some patients given high doses of IL-2 with or without lymphokine activated killer (LAK) cells or tumor infiltrating lymphocytes (TIL's) (Rosenberg, S.A. et al. (1998) *J Natl Cancer Inst* 90:1894). 3. More recently, a number of melanoma specific and associated tumor antigens have been cloned (Van den Eynde, B. et al. (1997) *Curr Opin Immunol* 9:684). The availability of these reagents has given hope that specific vaccines may be developed to enhance the ability of tumor specific T cells to eliminate melanoma cells. These antigens can be administered in a variety of delivery vehicles, and the most effective dosing regimen for optimization of an anti-tumor response is not presently clear.

Conventional vaccines for many infectious diseases have shown that a primary infectious challenge can induce an effective protective memory immune response. A critical event required to initiate an immune response, even in the presence of circulating tumor reactive T cells, is that tumor antigens gain entry

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into the secondary lymphoid structures of the spleen and lymph nodes (Zinkernagel, R.M. et al. (1997) *Immunol Rev* 156:199). Melanoma that develops locally in the skin is hidden from the immune system as long as the tumor antigens do not reach the secondary lymphoid organs. The tumor may then  
5 grow to such a large mass that, by the time an immune response is triggered, it is rapidly overwhelmed by the tumor (Moskophiis, D. et al. (1993) *Nature* 362:758). Alternatively, melanoma cells that break off and traffic to the local lymph node may not be able to trigger an immune response because they are poor antigen presenting cells, i.e., they do not express high levels of HLA-class I  
10 molecules (Ferrone, S. (1995) *Immunology Today* 61:487) or co-stimulatory molecules, such as members of the B7 family (Bluestone, J.A. et al. (1995) *Immunity* 2:555). Again, the tumor may reach a tolerizing size before an immune response is initiated. A successful vaccination strategy in patients with deep, but not metastatic, melanoma should both increase the number of tumor reactive T  
15 cells and activate them so that they can traffic to the periphery and exert their cytotoxic effector function.

gp100 is normally found in melanosomes and expressed in melanocytes, retinal cells, and other neural crest derivatives (Kawakami, Y. et al. (1997) *Int Rev Immunol* 14:173). The function of gp100 is currently unknown (Rosenberg, S.A. et al. (1998) *Nature Med* 4:321). By mass spectrometry, three immunodominant  
20 HLA-A2 binding gp100 peptides have been identified: g9-154 (amino acids 154-162), g9-209 (amino acids 209-217); and g9-280 (amino acids 280-288) (Kawakami, Y. et al. (1995) *J Immunol* 154:3961). Notably, two of these peptides have been synthetically altered so as to induce a more vigorous immune response in the  
25 original T cell clone: the threonine at position 2 in g9-209 was changed to a methionine, and the alanine residue at position 9 in g9-280 was changed to a valine (Parkhurst, M.R. et al. (1996) *J Immunol* 157:2539). These changes increase the binding affinity of the peptides to the HLA-A2 molecule without changing the epitopes recognized by the T cell receptor (TCR). Rosenberg et al have  
30 already successfully immunized melanoma patients with one of these modified

peptides and achieved objective clinical responses in some patients (Rosenberg, S.A. et al. (1998) *Nature Med* 4:321).

#### **SUMMARY OF THE INVENTION**

The present inventors have developed a molecule useful in modulating the immune system. In particular, they have found that a nucleotide sequence for a full gp100 which has been modified is useful in modulating the immune system. The isolated nucleic acid sequence is referred herein as gp100M and the corresponding protein is referred to herein as gp100M. Preferably the molecule (i.e. protein or nucleic acid) or immunogenic fragments of the protein may be used to prime and boost the immune system of an animal.

Accordingly, the present invention provides an isolated nucleic acid molecule comprising a sequence encoding a modified gp100 protein, preferably the gp100M protein. Preferably, a modified gp100 protein according to the present invention is one wherein the modification results in at least one amino acid modification of the said gp100, although any other modification capable of modifying gp100 so as to enable it to modify the immune system is within the scope of the present invention.

The nucleic acid sequence of gp100M is shown in Figure 1 and is also known herein as SEQ.ID.NO.1. The corresponding amino acid sequence encoded by the nucleic acid sequence of gp100M is shown in Figure 2 which is referred to herein as SEQ.ID.NO.2.

Accordingly, in one embodiment of the invention, an isolated nucleic acid molecule is provided having a sequence as shown in Figure 1 (SEQ.ID.NO.1).

Preferably, the purified and isolated nucleic acid molecule comprises:

- (a) a nucleic acid sequence as shown in SEQ.ID.NO.1 wherein T can also be U;
- (b) nucleic acid sequences complementary to (a);
- (c) nucleic acid sequences which are homologous to (a) or (b);

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(d) a fragment of (a) to (c) that is at least 15 bases, preferably 20 to 30 bases, and which will hybridize to (a) to (d) under stringent hybridization conditions; or

(e) a nucleic acid molecule differing from any of the nucleic acids of (a) to  
5 (c) in codon sequences due to the degeneracy of the genetic code.

The invention further includes an isolated gp100M protein and/or immunogenic fragments thereof encoded by a nucleic acid molecule of the invention. In a preferred embodiment the gp100M has the amino acid as shown in Figure 2 (SEQ.ID.NO.2).

10 Preferred embodiments of these fragments include a fragment having an amino acid sequence according to SEQ.ID.NO.124 or according to SEQ.ID.NO.125.

The invention further provides a method of modulating an animal's immune system comprising administering, to an animal in need thereof, an  
15 effective amount of a gp100 or gp100 which has been modified to provide a molecule which modulates the immune system. Preferably the modified gp100 or gp100 is gp100M or gp100M, respectively, most preferably the modified gp100 or gp100 have the sequences of SEQ. ID. NO 1 and 2 respectively.

The invention further provides a method of modulating an animal's  
20 immune system comprising administering to an animal in need thereof, an effective amount of a vector into which has been inserted a gp100 which has been modified to provide a molecule which modulates the immune system, preferably the vector is viral, preferably the virus is an adenovirus, alphavirus or poxvirus. More preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox,  
25 TROVAC, ALVAC, NYVAC or MVA, preferably ALVAC. .

According to another embodiment of the present invention the modified gp100 or gp100 is administered with a second agent, preferably a lymphokine, cytokine, or co-stimulatory molecule such as a member of the B7 family of molecules, preferably the cytokine is GM-CSF, IL-2, IL-12, TNF or  
30 IFN $\gamma$ 1.

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In one embodiment, the present invention provides a method of stimulating an animal's immune system comprising administering to an animal in need thereof, an effective amount of a gp100 or *gp100* which has been modified to provide a molecule which stimulates the immune system, preferably gp100M or *gp100M*, respectively, most preferably the modified gp100 or *gp100* have the sequences of SEQ. ID. NO 1 and 2 respectively

The present invention also provides a method of enhancing the efficacy of a gene vaccine in the treatment of an animal comprising administering an effective amount of a *gp100* which has been modified to provide a molecule which stimulates the immune system, preferably *gp100M*. According to a preferred embodiment, the animal has cancer.

According to a further embodiment of the present invention, there is provided a composition for modulating an animal's immune system comprising an effective amount of a gp100 or *gp100* which has been modified to provide a molecule which stimulates the immune system, preferably gp100M or *gp100M* respectively, in a pharmaceutically acceptable diluent or carrier, most preferably the modified gp100 or *gp100* have the sequences of SEQ. ID. NO 1 and 2 respectively.

According to another aspect of the present invention, provided are methods for prophylactic or therapeutic uses involving a nucleic acid sequence encoding a modified gp100, preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2.

According to a further aspect of the present invention, there is provided a melanoma vaccine comprising a nucleic acid sequence encoding a modified gp100 for preventing or treating cancer preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2..

According to a further aspect of the present invention, there is provided a melanoma vaccine comprising a nucleic acid sequence encoding a modified gp100 for preventing or treating melanoma preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2..

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According to yet another aspect of the present invention, there is provided a modified gp100 protein sequence which is modified to increase its immunogenicity or to enhance its induction of an anti-melanoma immune response by enhancing the binding to MHC molecules, for use in a prophylactic or therapeutic methods described herein.

According to another aspect of the present invention, there is provided a vaccine comprising a modified gp100 nucleic acid sequence or its corresponding protein capable of eliciting the production of antibodies in a mammal to corresponding antigens preferably *gp100M* or *gp100M*, respectively, more preferably a *gp100M* having a nucleic acid sequence according to SEQ ID NO. 1 and a *gp100M* having an amino acid sequence according to SEQ ID NO. 2.

According to another aspect, the present invention relates to an antigenic, immunological or vaccine composition or a therapeutic composition for inducing an antigenic or immunological response in a host animal inoculated with the composition, said vaccine including a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety.

In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further contains DNA from a heterologous source in a nonessential region of the virus genome.

According to another embodiment of the invention there is provided a recombinant virus comprising a virus into which is inserted a nucleic acid according to the invention wherein the nucleic acid encodes for a polypeptide,



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the recombinant virus causing the expression of the polypeptide in an infected cell.

In another embodiment, there is provided a recombinant virus into which is inserted a nucleic acid according to the present invention wherein the nucleic acid encodes for a modified gp100 polypeptide, wherein cells infected with the said recombinant virus are capable of eliciting an immune response directly against a member selected from the group consisting of:

- (1) the polypeptide;
- (2) a fragment of the polypeptide;
- (3) a cell expressing the polypeptide or a fragment thereof; or
- (4) cells binding the protein or fragment thereof, preferably the virus is adenovirus, alphavirus, or poxvirus, preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA, preferably the virus is ALVAC.

In an embodiment such recombinant virus' are part of a composition comprising the particular recombinant virus and a pharmaceutically acceptable diluent or carrier.

The invention in yet a further aspect relates to the product of expression of a recombinant virus including a nucleic acid encoding a modified gp100, preferably the virus is an adenovirus, alphavirus or poxvirus, more preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA, preferably ALVAC, and uses therefor, such as to form antigenic, immunological or vaccine compositions for treatment, prevention, diagnosis or testing; and, to DNA from the recombinant poxvirus which is useful in constructing DNA probes and PCR primers.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only. Various changes and modifications within the spirit and scope

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of the invention will become apparent to those skilled in the art from this detailed description.

#### **DESCRIPTION OF THE DRAWINGS**

5       The invention will be better understood with reference to the drawing in which:

Figure 1 shows the nucleic acid sequence of *gp100M* cDNA.

Figure 2 shows the deduced amino acid sequence for the *gp100M* protein.

10       Figure 3 shows the nucleic acid sequence of C5H6gp100M, the H6 promoted human *gp100M* insertion cassette.

Figure 4 shows a schematic representation of the ALVAC(2)-*gp100M*(vCP1584) genome.

Figure 5 shows the nucleotide sequence of the oligonucleotide primers used to sequence pBS/1584.

15       Figure 6 shows immunoprecipitate results from uninfected HeLa cells or cells infected with either ALVAC parental virus, ALVAC-*gp100*, or ALVAC (2)-*gp100M*.

20       Figure 7 shows a Western blot of HeLa cells infected with one of ALVAC parental virus, ALVAC-*gp100*, or ALVAC (2)-*gp100M*, illustrating expression of full length *gp100* in ALVAC-*gp100*, or ALVAC (2)-*gp100M* infected cells.

Figure 8 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving intranodal injection of the tumor antigen.

25       Figure 9 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving intranodal injection of the tumor antigen.

Figure 10 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving subcutaneous injection of the tumor antigen.

Figure 11 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving subcutaneous injection of the tumor antigen.

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Figure 12 is a graph showing the antibody response after a regiment of intranodal (group 2) and subcutaneous (group 3) administration of ALVAC-modified gp100/modified gp100 peptide immunogens.

#### **DETAILED DESCRIPTION OF THE INVENTION**

5 As already mentioned, the present inventors have developed a molecule useful in modulating the immune system of an animal. In particular, they have found that a nucleotide sequence for a full gp100, which has been modified, is useful in modulating the immune system. The term "animal" as used herein includes all members of the animal kingdom including mammals, preferably  
10 humans.

#### **I. NUCLEIC ACID MOLECULES OF THE INVENTION**

As mentioned above, the inventors have isolated and characterized the gene (*gp100M*) and its gene product (*gp100M*).

Broadly stated, the present invention provides an isolated nucleic acid  
15 molecule comprising a sequence encoding a protein with the activity of a gp100 which has been modified to provide a molecule which stimulates the immune system. As used herein a gp100 which has been modified to provide a molecule which stimulates the immune system includes those gp100 sequences with modified sequences at about amino acids 209 and/or at about 280 (as set out in  
20 Parkhurst, M.R., et al. J. Immunol. 157:2539-2548 (1996)) and/or immunogenic fragments thereof.

Accordingly, the present invention includes any isolated nucleic acid molecule encoding a modified gp100 capable of modulating the immune system. The term "isolated" refers to a nucleic acid substantially free of cellular material or  
25 culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded. In an embodiment of the invention, an isolated nucleic acid molecule is provided having a sequence as shown in Figure 1.

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Preferably, the purified and isolated nucleic acid molecule comprises:

- (a) a nucleic acid sequence as shown in Fig. 1, wherein T can also be U;
- (b) nucleic acid sequences complementary to (a);
- (c) nucleic acid sequences which are homologous to (a) or (b);
- 5 (d) a fragment of (a) to (c) that is at least 15 bases, preferably 20 to 30 bases, and which will hybridize to (a) to (c) under stringent hybridization conditions; or
- (e) a nucleic acid molecule differing from any of the nucleic acids of (a) to (c) in codon sequences due to the degeneracy of the genetic code.

10 It will be appreciated that the invention includes nucleic acid molecules encoding truncations of the proteins of the invention, and analogs and homologs of the proteins of the invention and truncations thereof, as described below. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a  
15 cDNA of the invention are encompassed by the invention.

Further, it will be appreciated that the invention includes nucleic acid molecules comprising nucleic acid sequences having substantial sequence homology with the nucleic acid sequence as shown in Figure 1 and fragments thereof. The term "sequences having substantial sequence homology" means  
20 those nucleic acid sequences which have slight or inconsequential sequence variations from these sequences, i.e., the sequences function in substantially the same manner to produce functionally equivalent proteins. The variations may be attributable to local mutations or structural modifications.

Generally, nucleic acid sequences having substantial homology include  
25 nucleic acid sequences having at least 70%, preferably 80-90% identity with the nucleic acid sequence as shown in Figure 1.

Another aspect of the invention provides a nucleic acid molecule, and fragments thereof having at least 15 bases, which hybridize to the nucleic acid molecules of the invention under hybridization conditions, preferably stringent  
30 hybridization conditions. Appropriate stringency conditions which promote

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DNA hybridization are known to those skilled in the art (for example, Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6). Hybridization procedures are also well known to those skilled in the art (for example, as described in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc. (1994), Silhavy *et al.*, Experiments with Gene Fusion, Cold Spring Harbor Laboratory Press (1984); Davis *et al.* Methods in Enzymol. 65:404 (1980)). Important parameters that can be considered for optimizing hybridization conditions are reflected in a formula that allows calculation of a critical value, the melting temperature above which two complementary DNA strands separate from each other (Davis *et al.* Methods in Enzymol. 65:404(1980)). This formula is as follows:  $T_m = 81.5 + 0.41 \times (\% \text{ G+C}) + 16.6 \log (\text{actual ion concentration}) - 0.63 \times (\% \text{ formamide}) - 600/\text{base number}$ . Under appropriate stringency conditions, hybridization temperature ( $T_h$ ) is approximately 20 to 40°C, 20 to 25°C, or, preferably 30 to 40°C below the calculated  $T_m$ . Those skilled in the art will understand that optimal temperature and salt conditions can be readily determined empirically in preliminary experiments using conventional procedures.

For example, stringent conditions can be achieved, both for pre-hybridizing and hybridizing incubations, (i) within 4 to 16 hours at 42°C, in 6 x SSC containing 50% formamide or (ii) within 4-16 hours at 65°C in an aqueous 6 x SSC solution (1 M NaCl, 0.1 M sodium citrate (pH 7.0)). Typically, hybridization experiments are performed at a temperature from 60 to 68°C, e.g. 65°C. At such a temperature, stringent hybridization conditions can be achieved in 6xSSC, preferably in 2xSSC or 1xSSC, more preferably in 0.5xSSC, 0.3xSSC or 0.1xSSC (in the absence of formamide). 1xSSC contains 0.15 M NaCl and 0.015 M sodium citrate.

For polynucleotides containing 30 to 600 nucleotides, the above formula is used and then is corrected by subtracting (600/polynucleotide size in base pairs). Stringency conditions are defined by a  $T_h$  that is 5 to 10°C below  $T_m$ .

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Hybridization conditions with oligonucleotides shorter than 20 to 30 bases do not exactly follow the rules set forth above. In such cases, the formula for calculating the  $T_m$  is as follows:  $T_m = 4 \times (G+C) + 2 \times (A+T)$ . For example, an 18 nucleotide fragment of 50% G+C would have an approximate  $T_m$  of 54°C.

5 Nucleic acid molecules from a modified *gp100* gene such as the *gp100M* gene can be isolated by preparing a labeled nucleic acid probe based on all or part of the nucleic acid sequence as shown in Figure 1, and using this labeled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). Nucleic acids isolated by screening of a cDNA or  
10 genomic DNA library can be sequenced by standard techniques.

Nucleic acid molecules of the invention can also be isolated by selectively amplifying a nucleic acid using the polymerase chain reaction (PCR) method and cDNA, genomic DNA or other source of DNA. It is possible to design synthetic oligonucleotide primers from the nucleic acid molecule as shown in Figure 1 for  
15 use in PCR. These synthetic oligonucleotide primers may also be further modified to incorporate specific changes from the normal nucleic acid sequence so as to be utilized for site directed mutagenesis. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an  
20 appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., (Biochemistry, 18, 5294-5299 (1979)). cDNA is then synthesized from the mRNA using reverse transcriptase  
25 (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a novel protein of the invention into an  
30 appropriate vector which allows for transcription of the cDNA to produce an

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RNA molecule which encodes the gp100M protein. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g., a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

- 5       A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., U.S. Patent No. 4,598,049; U.S. Patent No. 4,458,066; 10 and U.S. Patent Nos. 4,401,796 and 4,373,071).

- The initiation codon and untranslated sequences of the nucleic acid molecules of the invention may be determined using currently available computer software designed for the purpose, such as PC/Gene (IntelliGenetics Inc., Calif.). Regulatory elements can be identified using conventional 15 techniques. The function of the elements can be confirmed by using these elements to express a reporter gene which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures. In addition to identifying regulatory elements in DNA, such constructs may also be used to identify proteins interacting with the elements, 20 using techniques known in the art.

      The invention also provides nucleic acids encoding fusion proteins comprising a novel protein of the invention and a selected protein, or a selectable marker protein (see below).

## 25   II. METHODS OF EXPRESSING NUCLEIC ACID SEQUENCES OF THE INVENTION

- A polynucleotide molecule of the invention, containing RNA, DNA, or modifications or combinations thereof, can have various applications. For example, a polynucleide molecule can be used (i) in a process for producing the encoded polypeptide in a recombinant host system, (ii) in the construction of 30 vaccine vectors (such as poxviruses) used for modulating immune systems; (iii)

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as vaccine vectors which are further used in methods and compositions for preventing and/or treating melanoma, (iv) as a vaccine agent (as well as an RNA molecule), in a naked form or formulated with a delivery vehicle and, (v) as detection reagents/hybridization probes in molecular and/or therapeutic assays.

5 According to further aspects of the invention, there are provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or  
10 transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA  
15 molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system can be selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells),  
20 arthropods cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells), and plant cells. Preferably, a procaryotic host such as *E. coli* is used. Bacterial and eucaryotic cells are available from a number of different sources to those skilled in the art, *e.g.*, the American Type Culture Collection (ATCC; 10801 University Blvd., Manassas, VA 20110-2209, USA).

25 The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

The choice of the expression cassette will depend on the host system selected as well as the features desired for the expressed polypeptide. Typically,  
30 an expression cassette includes a promoter that is functional in the selected host



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system and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary; a region encoding a signal peptide (e.g., a lipidation signal peptide); a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide-encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region can be homologous or heterologous to the DNA molecule encoding the mature polypeptide and can be specific to the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters, (and signal peptide encoding regions) are widely known and available to those skilled in the art and include, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, Protein Eng. 4:843 (1991)), the promoter of the gene of bacteriophage T7 encoding RNA polymerase that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496), OspA lipidation signal peptide, and the RlpB lipidation signal peptide (Cagnon *et al.*, Protein Eng. 4:843 (1991)).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (e.g., plasmids or viral vectors) can be chosen from those described in Pouwels *et al.* (Cloning Vectors: A Laboratory Manual 1985, Supp. 1987). They can be purchased from various commercial sources.

Methods for transforming/transfecting host cells with expression vectors will depend on the host system selected as described in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc. (1994).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular

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compartment, is secreted/excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide can then be recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture.

5 Typically, the recombinant polypeptide can be purified by antibody-based affinity purification or by any other method that can be readily adapted by a person skilled in the art, such as by genetic fusion to a small affinity binding domain. Antibody-based affinity purification methods are also available for purifying a polypeptide of the invention. Antibodies useful for purifying by  
10 immunoaffinity the polypeptides of the invention can be obtained as described below.

According to further aspects of the invention, there are provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of  
15 an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention which involves culturing a procaryotic or  
20 eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention under conditions that allow expression of the DNA molecule of the invention, and recovering the encoded polypeptide or polypeptide derivative from the cell culture.

### **III. NOVEL PROTEINS OF THE INVENTION**

25 The invention further includes an isolated protein encoded by nucleic acid molecules of the invention. Within the context of the present invention, a protein of the invention may include various structural forms of the primary protein which retain biological activity. As used herein "modified gp100" or "modified gp100 protein", "gp100M" or "gp100M protein", means a gp100 which  
30 has been modified to provide a molecule which modulates the immune system.

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By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Both terms are used interchangeably in the present application.

The terms "modified gp100", "modified gp100 protein", "gp100M" or "gp100M protein" as used herein is intended to include analogs of a modified gp100 or gp100M, containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to a modified gp100M. Non-conserved substitutions involve replacing one or more amino acids with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the amino acid sequence of modified gp100, preferably gp100M. Amino acid insertions may consist of single amino acid residues or sequential amino acids.

Deletions may consist of the removal of one or more amino acids, or discrete portions (i.e. amino acids) from the gp100M amino acid sequence. The deleted amino acids may or may not be contiguous.

Also included in the expression "gp100M", "modified gp100", "modified gp100 protein" or "gp100M protein" as used herein are homologs of gp100M. Such homologs are proteins whose amino acid sequences are comprised of the amino acid sequences of gp100M regions from other sources whose coding nucleic acid sequences hybridize under stringent hybridization conditions (which conditions are known to those skilled in the art) with a nucleic acid probe used to obtain gp100M. It is anticipated that a protein comprising an amino acid sequence which is at least 72%, preferably 75 to 90% similar, with the amino acid sequence of gp100M will exhibit gp100M activity.

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As used herein the expressions "modified gp100", "modified gp100 protein", "gp100M", or "gp100M protein" also contemplate isoforms of the modified gp100, or gp100M protein. An isoform contains the same number and kinds of amino acids as the modified gp100 or gp100M, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein. Also included in the expression are other proteins which share similarities to a modified gp100 or gp100M.

Broadly stated, the present invention provides an isolated protein with activity equivalent to that of a modified gp100, preferably a gp100M protein. In a preferred embodiment of the invention, the protein has the amino acid sequence as shown in Figure 2.

In addition to full length amino acid sequences, the proteins of the present invention also include truncations of the protein and analogs and homologs of the protein and truncations thereof as described herein. Truncated proteins may comprise peptides of at least fifteen amino acid residues. Analogs of the protein having the amino acid sequence shown in Figure 2 and/or truncations thereof as described herein, may include, but are not limited to, an amino acid sequence containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the proteins of the invention with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent. Non-conserved substitutions involve replacing one or more amino acids of the amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the amino acid sequences shown in Figure 2. Amino acid insertions may consist of single amino acid residues or a range of sequential amino acids.

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Deletions may consist of the removal of one or more amino acids, or discrete portions from the amino acid sequence shown in Figure 2. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 100  
5 amino acids.

Analog of a protein of the invention may be prepared by introducing mutations in the nucleotide sequence encoding the protein. Mutations in nucleotide sequences constructed for expression of analogs of a protein of the invention must preserve the reading frame of the coding sequences.  
10 Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which could adversely affect translation of the receptor mRNA.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites  
15 enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site specific mutagenesis procedures may be employed to provide an altered gene having particular  
20 codons altered according to the substitution, deletion, or insertion required. Deletion or truncation of a protein of the invention may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are  
25 disclosed by Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989).

The proteins of the invention also include homologs of the amino acid sequence shown in Figure 2 and/or truncations thereof as described herein. Such homologs are proteins whose amino acid sequences are comprised of  
30 amino acid sequences whose coding nucleic acid sequences hybridize under

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stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a nucleic acid probe used to obtain a protein of the invention.

A homologous protein includes a protein with an amino acid sequence  
5 having at least 70%, preferably 80-90% identity with the amino acid sequence as shown in Figure 2. Homology is typically measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned to obtain the  
10 maximum degree of homology (*i.e.*, identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once the optimal alignment has been set up, the degree of homology (*i.e.*, identity) is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions. For example, sequence alignments may be  
15 performed using the ALIGN (Trademark) or GENALIGN (Trademark) computer programs (Intelligenetics Suite 5.4, Oxford Molecular). ALIGN uses the Needleman-Wunsch algorithm (Needleman and Wunsch, J. Mol. Biol. 48:443 (1970)) and its later modifications to locate regions of similarity between two sequences. Finding regions of maximum similarity between two sequences can  
20 be solved in a rigorous manner using the iterative matrix calculation of the Needleman and Wunsch 1997 algorithm. The analysis is restricted to regions with no internal deletions or insertions, joined by a minimum number of loop-outs or deletions. Sellers (J. Appl. Math (Siam) 26:787 (1974)) developed a true metric measure of the "distance" between sequences and Waterman et al.  
25 (Advan. Math 20:367 (1976)) extended this algorithm to include insertions and deletions of arbitrary length. Smith (J. Mol. Biol. 147:195 (1981)) improved the early algorithms to find the subsequences of maximum similarity. The algorithm has been used to analyze sequences as long as 5000 bases by dividing these sequences into segments of 200 to 400 bases, and then reassembling them into a  
30 final best match. This method of dividing the sequence and then reassembling it

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has proven quite robust. The algorithm permits the size of the segment to be specified which the program searches for similarities. The program then assembles the segments after checking overlaps of adjacent subsequences. The weighting of deletions and the relative size of overlaps may be controlled. The program displays the results to show the differences in closely related sequences. GENALIGN is a multiple alignment program. Up to 99 sequences using the Martinez/Regions (Sobel and Martinez, Nucleic Acid Res 14:363 (1985)) or Needleman-Wunsch (Needleman and Wunsch, J. Mol. Biol. 48:443 (1970)) method may be analyzed for alignment. GENALIGN places the sequences in an order that puts the most closely aligned sequence pairs adjacent to each other. A consensus sequence is displayed under the multiple sequence alignments. The sequences used in developing the consensus sequence file for use in other programs. GENALIGN allows the parameters of the search to be changed so that alternate alignments of the sequences can be formed.

These programs are used employing their default settings. The default settings are as follows:

	FastDB	
	AMINO-Res-length	= 2
	Deletion-weight	= 5.00
20	Length-factor	= 0
	Matching-weight	= 1.00
	NUCLEIC-Res-length	= 4
	Spread-factor	= 50
	Findseq	
25	<u>Search Parameters:</u>	
	Similarity matrix	Unitary
	K-tuple	4
	Mismatch penalty	1
	Joining Penalty	30
30	Randomization group length	0

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	Cutoff score	5
	<u>Alignment Parameters:</u>	
	Window size	32
	Gap penalty	1.00
5	Gap size penalty	0.33

The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as a protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same properties as a protein of the invention as described herein.

Polypeptides having a sequence homologous to the sequence of a modified gp100 such as that shown in Figure 2, include naturally-occurring allelic variants, as well as mutants or any other non-naturally occurring variants that are analogous in terms of antigenicity, to a polypeptide having a sequence as shown in Figure 2.

As is known in the art, an allelic variant is an alternate form of a polypeptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does not alter the biological function of the polypeptide. By "biological function" is meant the function of the polypeptide in the cells in which it naturally occurs, even if the function is not necessary for the growth or survival of the cells. For example, the biological function of a porin is to allow the entry into cells of compounds present in the extracellular medium. The biological function is distinct from the antigenic function. A polypeptide can have more than one biological function.

Allelic variants are very common in nature. For example, a bacterial species (i.e. *C. pneumoniae*) is usually represented by a variety of strains that differ from each other by minor allelic variations. Indeed, a polypeptide that fulfills the same biological function in different strains can have an amino acid sequence that is not identical in each of the strains. Such an allelic variation may be equally reflected at the polynucleotide level.



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The present invention also includes a protein of the invention conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion proteins. Additionally, immunogenic portions and/or fragments of a modified gp100 of the invention are within the scope of the invention.

5       An example of fusion polypeptides included in this invention includes a polypeptide or polypeptide derivative of the invention fused to a polypeptide having adjuvant activity (such as, for example, subunit B of either cholera toxin or *E. coli* heat-labile toxin). Several possibilities exist for achieving fusion. First, the polypeptide of the invention can be fused to the N-, or preferably, to the C-  
10       terminal end of the polypeptide having adjuvant activity. Second, a polypeptide fragment of the invention can be fused within the amino acid sequence of the polypeptide having adjuvant activity.

      The proteins of the invention (including truncations, analogs, etc.) may be prepared using recombinant DNA methods. Accordingly, the nucleic acid  
15       molecules of the present invention having a sequence which encodes a protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-  
20       associated viruses), so long as the vector is compatible with the host cell used. The expression "vectors are suitable for transformation of a host cell" is defined as meaning that the expression vectors contain a nucleic acid molecule of the invention and attendant regulatory sequences selected on the basis of the host cells to be used for expression, said regulatory sequence being operatively linked  
25       to the nucleic acid molecule. "Operatively linked" is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

      The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a  
30       fragment thereof, and the necessary regulatory sequences for the transcription

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and translation of the inserted nucleotide-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, or viral genes. (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990)). Selection of appropriate regulatory sequences is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include the following: a transcriptional promoter and enhancer, or RNA polymerase binding sequence, or a ribosomal binding sequence (including a translation initiation signal). Additionally, depending on the host cell chosen and the vector employed, other sequences (such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription) may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may also be supplied by the *gp100/gp100M* gene and/or its flanking regions in addition to the above mentioned regulatory gene sequence(s).

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to a nucleotide sequence comprising the nucleotides as shown in Figure 1. Regulatory sequences operatively linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin (which confer resistance to certain drugs),  $\beta$ -galactosidase,

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chloramphenicol acetyltransferase, or firefly luciferase. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. As is known to one skilled in the art, cells that have incorporated the selectable marker gene will survive, while cells which do not have any such incorporated detectable marker will die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention. It will also be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and/or aids in the purification of a target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The term "transformed host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming

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and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic  
5 host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

10 The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, J. Am. Chem. Assoc. 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, Methods of Organic Chemistry (1987), (ed. E. Wansch) Vol. 15, pts. I and II, Thieme, Stuttgart).

#### 15 IV. VACCINES

The present invention includes a vaccine for modulating an animal's immune system wherein the immunogen is an effective amount of a *gp100* or modified *gp100*, and/or immunogenic fragments thereof, preferably in admixture with a suitable diluent or carrier.

20 Accordingly, the present invention also includes a method of modulating an animal's immune response comprising administering an effective amount of a *gp100* or modified *gp100* and/or immunogenic fragments thereof, preferably in admixture with a suitable diluent or carrier, to an animal in need thereof.

25 The vaccines of the present invention may additionally contain suitable diluents, adjuvants and/or carriers. Preferably, the vaccines contain one or more other adjuvants which can further enhance the immunogenicity of the vaccine *in vivo*. These other one or more adjuvants may be selected from many known adjuvants in the art including, for example, the lipid-A portion of the LPS from  
30 gram negative bacteria (endotoxin), trehalose dimycolate of mycobacteria, the

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phospholipid lysolecithin, dimethylditadecyl ammonium bromide (DDA), certain linear polyoxypropylene-polyoxyethylene (POP-POE) block polymers, aluminum hydroxide (and other aluminum compounds), and liposomes (see below).

5 Another preferred adjuvant/immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 9602555; European  
10 Patent EP 468520; Davies et al. (1998) *J. Immunol.* 160:87; McCluskie and Davis (1998) *J. Immunol.* 161:4463). In a number of studies, synthetic oligonucleotides derived from BCG gene sequences have also been shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo; Krieg, (1995) *Nature* 374:546). Detailed analyses of immunostimulatory oligonucleotide  
15 sequences has demonstrated that the CG motif must be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. (For example, the immunostimulatory sequence is often: purine, purine, C, G, pyrimidine, pyrimidine, wherein the CG motif is not methylated; however other unmethylated CpG sequences are known to be  
20 immunostimulatory and as such may also be used in the present invention.)

The vaccines may also include as adjuvants cytokines that are known to enhance immune responses (including GM-CSF, IL-2, IL-12, TNF and IFN $\gamma$ ), co-stimulatory molecules (such of those of the B7 family) and/or other lymphokines. The vaccine may also contain preservatives such as sodium azide,  
25 thimersol, beta propiolactone, and binary ethyleneimine.

The vaccine compositions of the invention are suitable for administration to subjects in a biologically compatible form *in vivo*. The expression "biologically compatible form suitable for administration in vivo" as used herein means a form of the substance to be administered in which any toxic effects are  
30 outweighed by the therapeutic effects.

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The dose of the vaccine may vary according to factors such as the disease state, age, sex, and weight of the , and the ability of the vaccine to elicit a desired response in the animal. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. The dose of the vaccine may also be varied to provide optimum preventative dose response depending upon the circumstances.

The vaccines may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, intranodal etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications.

As such, in one aspect of this invention, there are provided (i) a vaccine containing a modified gp100 (or immunogenic fragment thereof) of the invention; (ii) a composition of matter containing a gp100 (or immunogenic fragment thereof) of the invention, together with a diluent or carrier; (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a modified gp100 (or immunogenic fragment thereof) of the invention; (iv) a method for inducing an immune response against a modified gp100 (or immunogenic fragment thereof) in a mammal (for example, a human; alternatively, the method can be used in veterinary application) which involves administering the mammal an immunogenically effective amount of a modified gp100 (or immunogenic fragment thereof) of the invention to elicit an immune response (for example, a protective or therapeutic immune response to modified gp100); (v) a method for preventing and/or treating melanoma which involves administering a prophylactic or therapeutic amount of a modified gp100 (or immunogenic fragment thereof) of the invention to an individual in need. Additionally, the invention encompasses the use of a modified gp100 (or immunogenic fragment thereof) of the invention in the preparation of a medicament for preventing and/or treating of melanoma.

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A vaccine of the invention may contain a nucleic acid molecule encoding a modified gp100 protein of the invention. Such vaccines are referred to as nucleic acid vaccines but are also termed genetic vaccines, polynucleotide vaccines or DNA vaccines, all of which are within the scope of the present invention. In such an embodiment, the modified gp100 protein is produced *in vivo* in the host animal. The vaccines containing nucleic acids may be delivered using a suitable vector (ie. "vaccine vector") including, for example, retroviral vectors, alphaviral, adenoviral vectors, poxviral vectors, other viral vectors, bacterial DNA, plasmids, or free/naked DNA.

Accordingly, in additional aspects of the invention, there are provided (i) a vaccine vector containing a DNA molecule of the invention placed under the control of elements required for expression; (ii) a composition of matter containing a vaccine vector of the invention, together with a diluent or carrier; (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a vaccine vector of the invention; (iv) a method for inducing an immune response against modified gp100 in a mammal (for example, a human; alternatively, the method can be used in veterinary applications) which involves administering to the mammal an immunogenically effective amount of a vaccine vector of the invention to elicit an immune response, (for example, a protective or therapeutic immune response to modified gp100); (v) a method for preventing and/or treating melanoma which involves administering a prophylactic or therapeutic amount of a vaccine vector of the invention to an individual in need. Additionally, the invention encompasses the use of a vaccine vector of the invention in the preparation of a medicament for preventing and/or treating melanoma.

*i Vaccine Vector*

The vaccine vector may be a poxvirus or other viral vector, bacterial DNA, plasmid or a free/naked DNA. Preferably the vaccine vector is incapable of integration in recipient animal cells. The elements for expression from said

vaccine vector may include a promoter suitable for expression in recipient animal cells.

Live vaccine vectors available in the art include viral vectors such as alphaviruses, adenoviruses and poxviruses as well as bacterial vectors (for example, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille Calmette Guérin (BCG), and *Streptococcus*).

An example of an adenovirus vector, as well as a method for constructing an adenovirus vector capable of expressing a DNA molecule of the invention, are described in U.S. Patent No. 4,920,209 (incorporated herein by reference). Poxvirus vectors that can be used include, for example, fowlpox, vaccinia and canary pox virus (as described in U.S. Patent No. 5,766,599 and U.S. Patent No. 5,364,773, U.S. Patent No. 5,756,103, (ALVAC(2), U.S. Patent No. 5,990,091, U.S. Patent No. 6,004,777); Poxvirus vectors capable of expressing a nucleic acid of the invention can be obtained by homologous recombination as is known to one skilled in the art so that the polynucleotide of the invention is inserted in the viral genome under appropriate conditions for expression in mammalian cells (see below).

In one preferred aspect the engineered poxvirus vector is ALVAC (which has been derived from canarypox virus). ALVAC does not productively replicate in non-avian hosts, a characteristic thought to improve its safety profile.

ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al., *Viol.* 188:217 (1992)) (U.S. Patent No. 5,756,103). ALVAC has some general properties which are the same as some general properties of Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors (Tartaglia, J. et al, In AIDS Research Reviews (vol. 3), Koff, W., Wong-Staal, F., Kenedy, R.C. (eds), Marcel Dekker NY, pp. 361-378 (1993); Tartaglia, J. et al. *J. Virol.* 67:2370 (1993)). For instance, mice immunized with an ALVAC recombinant expressing the rabies virus glycoprotein were protected from lethal challenge with rabies virus



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(Tartaglia, J. et al. (1992), *Virology* 188:217) demonstrating the potential for ALVAC as a vaccine vector. ALVAC-based recombinants have also proven efficacious in dogs challenged with canine distemper virus (Taylor, J. et al., (1992), *Virology* 187:321) and rabies virus (Perkus, M.E. et al., In *Combined Vaccines and Simultaneous Administration: Current Issues and Perspective*, Annals of New York Academy of Sciences (1994)), in cats challenged with feline leukemia virus (Tartaglia, J. et al. *J. Virol.* 67:2370 (1993)), and in horses challenged with equine influenza virus (Taylor, J. et al., In *Proceedings of the Third International Symposium on Avian Influenza*, Univ. of Wisconsin-Madison, Madison, Wisconsin, pp. 331-335 (1993)).

ALVAC (2) is a second-generation ALVAC vector in which vaccinia transcription elements E3L and K3L have been inserted within the C6 locus (U.S. 5,990,091; U.S. 6,004,777). The E3L encodes a protein capable of specifically binding to dsRNA. The K3L ORF has significant homology to E1F-2. Within ALVAC (2) the E3L gene is under the transcriptional control of its natural promoter, whereas K3L has been placed under the control of the early/late vaccine H6 promoter. The E3L and K3L genes act to inhibit PKR activity in cells infected with ALVAC (II), allowing enhancement of the level and persistence of foreign gene expression.

Additional vaccine vector systems involve the use of naturally host-restricted poxviruses. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. Vectors derived from fowlpox have been generated (i.e., TROVAC, see U.S. Patent No. 5,766,599). Replication of the avipox viruses is limited to avian species (Matthews, *Inter Virol.* 17:42 (1982)) and there are no reports in the literature of avipoxvirus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipoxvirus based vaccine vectors in veterinary and human applications an attractive proposition.

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FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant. After inoculation of the recombinant into chickens and turkeys, an immune response was induced which  
5 was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor, J. et al., (1988) Vaccine 6:504). FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor, J. et al. (1990) J. Virol. 64:1441; Edbauer, C. et al., (1990) Virology 179:901).

10 Highly attenuated strain of vaccines, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Patent No. 5,185,146.

Other attenuated poxvirus vectors have been prepared by genetic modifications of wild type strains of virus. The NYVAC vector, for example, is  
15 derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia, J. et al. (1992) Virology 188:217) and has proven useful as a recombinant vector in eliciting a protective immune response against an expressed foreign antigen (U.S. 5,364,773). The TROVAC vector provides yet another example of an attenuated poxvirus which may be used  
20 (U.S. 5,766,599).

Recombinant poxviruses can be constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus (described in U.S. Patent Nos. 4,769,330; 4,744,848; 4,603,112; 5,100,587; and 5,179,993). Clearly, based on  
25 the attenuation profiles of the NYVAC, ALVAC, and TROVAC vectors and their demonstrated ability to elicit both humoral and cellular immunological responses to extrinsic immunogens (Tartaglia, J. et al, In AIDS Research Reviews (vol.3), Koff, W., Wong-Staal, F. and Kenedy, R.C. (eds), Marcel Dekker NY, pp. 361-378 (1993); Tartaglia, J. et al. (1993) J. Virol. 67:2370;; Taylor, J. et al., (1992) Virology  
30 187:321), such recombinant viruses offer a distinct advantage over previously

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described vaccinia-based recombinant viruses. It can thus be appreciated that provision of a modified gp100 recombinant poxvirus, and of compositions and products therefrom (particularly ALVAC-modified gp100 recombinants and compositions and products therefrom) would be a highly desirable advance over the current state of technology.

Plasmids and/or free/naked nucleic acids (i.e. polynucleotides (DNA or RNA)) of the invention can also be administered as vaccine vectors to an animal for vaccine (e.g., therapeutic or prophylactic) purpose (US Patent No. 5589466; McDonnell and Askari, NEJM 334:42-45 (1996); Kowalczyk and Ertl, Cell Mol. Life Sci. 55:751-770 (1999)). When a DNA molecule of the invention is used, it can be in a free/naked or plasmid form. Typically it is a form that is unable to replicate in a mammalian cell and unable to integrate in the mammalian genome. The DNA molecule is also typically placed under the control of a promoter suitable for expression in a mammalian cell. The promoter can function ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter. The desmin promoter is tissue-specific and drives expression in muscle cells. More generally, useful vectors have been described (i.e., WO 94/21797).

For DNA/RNA vaccination, the polynucleotide of the invention can encode a precursor or mature form of the modified gp100 or immunogenic fragment thereof. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

Standard techniques of molecular biology for preparing and purifying polynucleotides can be used in the preparation of polynucleotide aspects of the invention. For use as a vaccine, a polynucleotide of the invention can be formulated according to various methods known to those who are skilled in the art.

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First, a polynucleotide can be used in a naked/free form, free of any delivery vehicles (such as anionic liposomes, cationic lipids, microparticles, (e.g., gold microparticles), precipitating agents (e.g., calcium phosphate)) or any other transfection-facilitating agent. In this case the polynucleotide can be simply  
5 diluted in a physiologically acceptable solution (such as sterile saline or sterile buffered saline) with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength (such as provided by a sucrose solution (e.g., a solution containing 20% sucrose))

Alternatively, a polynucleotide can be associated with agents that assist  
10 in cellular uptake. It can be, *i.a.*, (i) complemented with a chemical agent that modifies the cellular permeability (such as bupivacaine; see, for example, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

Cationic lipids are well known in the art and are commonly used for  
15 gene delivery. Such lipids include Lipofectin( also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio) propane). DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl  
20 aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such as DOPE (dioleoyl phosphatidylethanolamine), as, for example, described in WO 90/11092.

25 Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, for example, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, *i.e.*, spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-

permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

Gold or tungsten microparticles can also be used for gene delivery (as described in WO 91/359 and WO 93/17706). In this case, the microparticle-coated polynucleotides can be injected *via* intradermal or intraepidermal routes using a  
5 needleless injection device ("gene gun"), such as those described, for example, in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263.

Anionic and neutral liposomes are also well-known in the art (see, for example, *Liposomes: A Practical Approach*, RPC New Ed, IRL Press (1990), for a  
10 detailed description of methods for making liposomes) and are useful for delivering a large range of products, including polynucleotides.

A vaccine vector of the invention can also express a cytokine (for example, such as interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte-macrophage colony stimulating factor (GM-CSF)) and/or co-stimulatory  
15 molecules (for example, such as the B7 family of molecules) and/or other lymphokines that enhance the immune response. Thus, a vaccine vector can include an additional DNA sequence encoding, for example, a cytokine and/or lymphokine and/or co-stimulatory molecule placed under the control of suitable elements required for expression in an animal cell.

20 Alternatively, a composition of the invention can include several vaccine vectors each being capable of expressing an aspect of the invention (i.e., polypeptide derivative of the invention, cytokine and/or lymphokine and/or costimulatory molecules) a polypeptide or derivative of the invention.

*ii Mode of Administration*

25 In vaccination methods for treating or preventing cancer in an animal, a vaccine vector of the invention can be administered by any conventional route in use in the vaccine field as is known to one skilled in the art. This may include, for example, administration to a mucosal (e.g., ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) surface, via the parenteral  
30 (e.g., subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal)

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route or intranodally. Preferred routes depend upon the choice of the vaccine vector. The administration can be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters understood by skilled artisans such as the vaccine vector itself, the route of administration  
5 and the condition of the animal to be vaccinated (weight, age and the like).

The administration of the vaccine or immunogen of the present invention may be for either a prophylactic or therapeutic purpose. When provided prophylactically, the immunogen is provided in advance of any evidence or in advance of any symptom due to melanoma, or in patients  
10 rendered free of disease by conventional therapies but at significant risk for reoccurrence. The prophylactic administration of the immunogen serves to prevent or attenuate melanoma in a mammal. When provided therapeutically, the immunogen is provided at (or after) the onset of the disease or at the onset of any symptom of the disease. The therapeutic administration of the  
15 immunogen serves to attenuate the disease.

A particularly preferred vaccination method encompasses a prime boost protocol. Recent studies have indicated that this protocol, whereby immunization with a poxvirus recombinant expressing a foreign gene product (or other nucleic acid encoding for a gene product) is followed by a boost using a  
20 purified subunit preparation form of that gene product (or nucleic acid coding therefor), elicits an enhanced immune response relative to the response elicited with either product alone. Accordingly, it is within the scope of the present invention to use a modified gp100 in a prime-boost protocol. Examples of methodologies teaching prime-boost protocol are described in WO 98/58956,  
25 WO 00/00216, WO 98/56919, WO 97/39771, and WO 98/58956 which are incorporated herein by reference.

The amount of naked/free DNA to be used in a vaccine recipient generally depends on the strength of the promoter used in the DNA construct, the immunogenicity of the expressed gene product, the condition of the animal  
30 intended for administration (i.e. the weight, age, and general health of the

animal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose from about 1 µg to about 1 mg, preferably, from about 10 µg to about 800 µg and, more preferably, from about 25 µg to about 250 µg, can be administered to human adults. The administration  
5 can be achieved in a single dose, repeated at intervals, or incorporated into prime boost protocols (as previously described).

*iii. Oral Vaccines*

Non-toxicogenic *Vibrio cholerae* mutant strains that are useful as a live oral vaccine are described, for example, in US Patent No. 4,882,278 (disclosing a strain in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted so that no functional cholerae toxin is produced);  
10 WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA* mutations); and WO 94/1533 (deletion mutant lacking functional *ctxA* and *attRS1* DNA sequences).  
15 These strains can be genetically engineered to express heterologous antigens, as described in WO 94/19482. (All of the aforementioned issued patent/patent applications are incorporated herein by reference.) An effective vaccine dose of a *Vibrio cholerae* strain capable of expressing a polypeptide or polypeptide derivative encoded by a DNA molecule of the invention can contain, for  
20 example, about  $1 \times 10^5$  to about  $1 \times 10^9$ , preferably about  $1 \times 10^6$  to about  $1 \times 10^8$  viable bacteria in an appropriate volume for the selected route of administration. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

Attenuated *Salmonella typhimurium* strains, genetically engineered for  
25 recombinant expression of heterologous antigens or not, and their use as oral vaccines are described, for example, in WO 92/11361. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

As will be readily appreciated by those skilled in the art, other bacterial  
30 strains useful as vaccine vectors may also include *Shigella flexneri*, *Streptococcus*

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gordonii, and Bacille Calmette Guerin (as described in WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376; all of which are incorporated herein by reference).

In bacterial vectors, a polynucleotide of the invention may be inserted into the bacterial genome, can remain in a free state or carried on a plasmid. An adjuvant can also be added to a composition containing a vaccine bacterial vector. A number of adjuvants are known to those skilled in the art. Suitable adjuvants can readily be selected by those skilled in the art.

#### **V. COMPOSITIONS**

A modified gp100 protein and gene, including the gp100M gene (*gp100M*) and gp100M protein as well as the substances identified using the methods described herein, including vaccines, may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to animals in need thereof. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention, or an "effective amount", are defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result of "modulating an animal's immune system". A therapeutically effective amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the animal, and the ability of immunogen to elicit a desired response in the animal. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. "Modulating an animal's immune system" is defined as the ability to produce an immune response in a target animal. This response encompasses both cellular and humoral immune responses.



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The active substance may be administered in a convenient manner such as by injection (intradermal, intramuscular, subcutaneous, intravenous, intranodal etc.), or by oral administration, inhalation, transdermal application, or rectal administration, or any other route of administration that enables the modulation of an animal's immune system. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

One route of administration which is preferably used is that of intranodal injection of a modified gp100 protein/immunogenic fragment, or nucleic acid encoding said protein/fragment, or any of the compositions of the present invention.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences (1985), Mack Publishing Company, Easton, Pa., USA) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456.

The utility of the substances, antibodies, and compositions of the invention may be confirmed in experimental model systems.

The following non-limiting examples are illustrative of the present invention:

#### EXAMPLES

ALVAC(2)-gp100M (vCP1584) is a preparation of recombinant canarypox virus expressing a modified version of human melanoma antigen gp100.

## A. MOLECULAR PROPERTIES (IDENTITY)

### 1. Recipient characterization:

5 a. Parental organism:

Canarypox virus: Family-Poxviridae, Subfamily -Chordopoxviridae, Genus-Avipoxvirus. Canarypox virus is an enveloped virus containing a linear dsDNA genome of approximately 325 kbp. This virus productively replicates exclusively in avian species ((Tripathy D. Avian pox. In: Diseases of Poultry (9<sup>th</sup> edition: B.W. Calnek et al. Eds.) pp. 583-596).

10 b. Description of vector:

ALVAC(2) is a modified, attenuated canarypox virus (U.S. 5,990,091). The original strain of canarypox virus (Rentschler strain) was attenuated by 200 serial passages on primary chick embryo fibroblasts (CEFs). The attenuated virus  
15 was plaque isolated and designated ALVAC. To generate ALVAC(2), the ALVAC vector was modified by the insertion of two vaccinia virus coding sequences (E3L and K3L) to enhance the overall efficiency of viral mRNA translation. The E3L-specified gene product is a dsRNA binding protein and the K3L open reading frame (ORF) shares significant sequence similarity to the  
20 amino terminal portion of eIF-2( (; Beattie et al. Virology 183:419 (1991); Beattie et al. Virology 210:254 (1995). Both K3L and E3L are capable of inhibiting the activity of a cellular protein kinase (PKR) which, when activated by dsRNA, phosphorylates the translational initiation factor eIF-2a leading to an inhibition of initiation of mRNA translation. Results from several studies have substantiated  
25 this proposed mechanism by which the vaccinia K3L and E3L gene products lead to down regulation of PKR activity and enhanced virus-specific gene expression. (Tartaglia, J. et al. 11<sup>th</sup> Colloque des Cent Gardes, Elsevier Press (1997)).

c. Derivation of vector from parental organism:

The parental strain of canarypoxvirus (Rentschler strain) was isolated in  
30 Germany in 1970, and obtained by Institut Merieux in 1973. The virus was

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attenuated by 200 serial passages on primary chick embryo fibroblasts (CEFs). The attenuated virus was registered as a vaccine in France in 1975 under the name KANAPOX (ND). The virus was subjected to four successive plaque purifications under agarose and a single plaque isolate was selected and  
5 designated ALVAC.

The K3L coding sequence was synthesized by PCR amplification using a plasmid containing Copenhagen vaccinia *HindIII* K fragment as template and was placed under the control of the vaccinia H6 promoter. The E3L coding sequence and upstream regulatory element were obtained from a plasmid  
10 containing a clone of the *HindIII* E fragment from Copenhagen vaccinia. The H6 promoted K3L and the E3L sequences were inserted into a donor plasmid capable of directing their insertion into the C6 site of the parent vector according to the details set out in Example 1. Recombination was performed between the donor plasmid and ALVAC rescuing virus by *in vitro* recombination as  
15 previously described (Piccini *et al.*, Methods of Enzymol. 153:545 (1987)). Expression of E3L and K3L in the resultant recombinant virus, vCP1468, was confirmed and vCP1468 was designated ALVAC(2).

d. Cloning site:

The locus designated C5 was used for the insertion of the modified  
20 gp100 coding sequences into the ALVAC(2) vector. By virtue of the C5 locus existing within the extensive inverted terminal repetitions (ITRs) of the virus genome, insertion into this locus results in the occurrence of two copies of the inserted sequence. A schematic of the insertion site is shown in Figure 4. Presently, no function has been ascribed to the C5 encoded polypeptide nor does  
25 the deduced amino acid open reading frame encoded in this region share significant homology to any entry in the existing protein databases.

**2. Donor characterization**

a. Donor organism:

Plasmids pCDNA3-gp100 and PCR11-gp100 each containing the gene  
30 encoding human gp100 (melanoma rejection antigen) were used.

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b. Donor genes:(i) Expressed donor genes: human gp100 melanoma antigen.(ii) Promoter: Vaccinia virus early/late H6 promoter (PerkusM.E. *et al*, J. Virol. 63:3829 (1989)).5 c. Donor genes in the recombinant organism:

The coding sequences for a modified human gp100 have been inserted into the ALVAC(2) genome to express the modified gp100 antigen enhanced for the interaction of two CTL epitopes with HLA class I.

**EXAMPLE 1**10 Insertion of the vaccinia E3L/K3L coding sequences into the C6 site of ALVAC

The K3L coding sequences were synthesized by PCR amplification using pSD407 (containing Copenhagen vaccinia *Hind*III K fragment) as template. The oligonucleotides MPSYN 763 (5'-CCCTCT AGATCG CGATAT CCGTTA AGTTTG TATCGT AATGCT TGCATT TTGTTA TTCGT-3') (SEQ.ID.NO.109) and  
 15 MPSYN 764 (5'-CCCGAA TTCATA AAAATT ATTGAT GTCTACA-3') (SEQ.ID.NO.110) were used as primers for the PCR reaction. The approximately 325bp PCR fragment was digested with *Xba*I and *Eco*RI yielding a 315bp fragment. This 315bp fragment was purified by isolation from an agarose gel and ligated with *Xba*I and *Eco*RI digested pBSSK+ vector from Stratagene (La  
 20 Jolla, CA.). The nucleic acid sequence was confirmed. This plasmid was designated pBS 763/764. Digesting pBS 763/764 with *Nru*I and *Xho*I a 340bp fragment was isolated for cloning into the plasmid vector pMM154. PMM154 contains a cassette with the vaccinia H6 promoter controlling an irrelevant gene in the NYVAC tk insertion vector background. PMM154 was prepared by  
 25 digestion with *Nru*I (partially) and *Xho*I such that the 340bp fragment from pBS 763 / 764 containing the K3L gene could be directionally oriented next to the H6 promoter generating pMPTKH6K3L. The plasmid pMP42CPT containing the dominant selectable marker *Eco* gpt gene under the control of the Entomopox 42k promoter was digested with *Sma*I and *Bam*HI to yield a 0.7 Kbp 42k- *Ecogpt*  
 30 expression cassette. This 0.7 Kbp fragment was purified and ligated into *Sma*I and

*Bam*HI cut pMPTKH6K3L generating the plasmid pMPTKH6K3Lgpt. This plasmid was digested with *Xho*I generating a 1.2 Kbp fragment containing the H6/K3L and the 42k/EcoGpt expression cassette which was then gel purified. The 1.2 Kbp *Xho*I fragment was inserted into the *Xho*I site of the ALVAC C6 insertion plasmid pC6L generating pMPC6H6K3Lgpt.

The entire E3L gene is contained within a 2.3 Kbp *Eco*RI fragment isolated from pSD401VC which contained a clone of the *Hind*III E fragment from Copenhagen vaccinia. The 2.3 Kbp *Eco*RI fragment was then inserted into pMPC6H6K3Lgpt that had been partially digested with *Eco*RI generating the plasmid pMPC6H6K3E3gpt. The plasmid pMPC6H6K3E3gpt was digested with *Xho*I. The resulting 6.8 Kbp vector fragment was purified and self-ligated, resulting in the plasmid pMPC6E3. The plasmid pMPTKH6K3L was digested with *Psp*AI and the resulting 560bp fragment containing the H6 / K3L expression cassette was ligated into *Psp*AI digested pMPC6E3 resulting in the plasmid construct pMPC6H6K3E3. The plasmid pMPC6H6K3E3 contains the vaccinia H6/ K3L expression cassette and the vaccinia E3L gene with the endogenous promoter flanked by the ALVAC C6 insertion site sequences.

## **EXAMPLE 2**

### Genetic modifications of the donor genes:

Plasmid pCDNA3-gp100 was transformed into MN522 yielding plasmid pMEL gp100 #1. A generic C5 donor plasmid NVQH6C5LSP-18 was digested within the polylinker region with *Bam*HI, treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides SPC5PL1 (5'-GAT-CGT-CGA-CGA-GCT-CGA-ATT-CG-3') (SEQ.ID.NO.111) and SPC5PL2 (5'-GAT-CCG-AAT-TCG-AGC-TCG-TCG-AC-3') (SEQ.ID.NO.112) generating plasmid NVQH6MC5 #10. Oligonucleotides MELgp01 (5'-CCC-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GGA-TCT-GGT-GCT-AAA-AAG-3') (SEQ.ID.NO.113) and MELgp02 (5'-CCC-CTC-GAG-ATA-AAA-ATC-AGA-CCT-GCT-GCC-CAC-TGA-3') (SEQ.ID.NO.114) were used in PCR with plasmid pMEL gp100 #1 to generate a 2kb fragment containing part of the H6 promoter linked to the 5' end of the